



---

Year: 2018

---

## **Sevoflurane Protects Hepatocytes From Ischemic Injury by Reducing Reactive Oxygen Species Signaling of Hepatic Stellate Cells: Translational Findings Based on a Clinical Trial**

Beck-Schimmer, Beatrice ; Roth Z'graggen, Birgit ; Booy, Christa ; Köppel, Sabrina ; Spahn, Donat R ;  
Schl  pfer, Martin ; Schadde, Erik

**Abstract:** **BACKGROUND** Randomized controlled trials (RCTs) data demonstrate that sevoflurane postconditioning improves clinical outcomes of liver resection with inflow occlusion, presumably due to hepatocyte protection from ischemic injury. However, mechanisms remain unclear. This study examines liver biopsy samples obtained in an RCT of sevoflurane postconditioning to test the hypothesis that sevoflurane attenuates hepatocyte apoptosis. **METHODS** Messenger ribonucleic acid (mRNA) of pro- and antiapoptotic regulators Bax and B-cell lymphoma 2 (Bcl2) was examined in hepatic biopsies obtained during the RCT. Hepatic stellate cells (HSCs) and hepatocytes were exposed to hypoxia/reoxygenation (H/R) in vitro to evaluate the effect of sevoflurane postconditioning on apoptosis. The role of HSC as a potential apoptosis trigger in hepatocytes through the production of reactive oxygen species induced by H/R was explored by transferring supernatants from H/R-exposed HSC to hepatocytes as target cells. **RESULTS** In patients of the RCT, the Bax/Bcl2 mRNA ratio in liver tissue was markedly decreased in the sevoflurane arm ( $25\% \pm 21\%$  reduction;  $P = .001$ ). In vitro, H/R increased reactive oxygen species production in HSC by  $33\% \pm 16\%$  ( $P = .025$ ), while it was abolished in the presence of sevoflurane ( $P < .001$ ). In hepatocytes, caspase was minimally activated by H/R. However, incubation of hepatocytes with supernatants of HSC, previously exposed to H/R, increased caspase activity by  $28\% \pm 13\%$  ( $P < .001$ ). When exposed to supernatants from HSC undergoing sevoflurane postconditioning, caspase activation in hepatocytes was reduced by  $20\% \pm 9\%$  ( $P < .001$ ), similarly to the sevoflurane effect on the BAX/Bcl2 mRNA ratio in the liver samples. **CONCLUSIONS** The study shows that sevoflurane postconditioning affects apoptosis of hepatocytes after ischemia-reperfusion injury in patients. It also demonstrates that HSC may be the effector cells of sevoflurane protection.

DOI: <https://doi.org/10.1213/ANE.0000000000003692>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-158376>

Journal Article

Published Version

Originally published at:

Beck-Schimmer, Beatrice; Roth Z'graggen, Birgit; Booy, Christa; K  ppel, Sabrina; Spahn, Donat R; Schl  pfer, Martin; Schadde, Erik (2018). Sevoflurane Protects Hepatocytes From Ischemic Injury by Reducing Reactive Oxygen Species Signaling of Hepatic Stellate Cells: Translational Findings Based on a Clinical Trial. *Anesthesia and Analgesia*, 127(4):1058-1065.

DOI: <https://doi.org/10.1213/ANE.0000000000003692>

# Sevoflurane Protects Hepatocytes From Ischemic Injury by Reducing Reactive Oxygen Species Signaling of Hepatic Stellate Cells: Translational Findings Based on a Clinical Trial

Beatrice Beck-Schimmer, MD,\*†‡ Birgit Roth Z'graggen, PhD,† Christa Booy,†  
Sabrina Köppel, MMed,\* Donat R. Spahn, MD,\* Martin Schläpfer, MD, MSc,\*† and Erik Schädde, MD†§||

**BACKGROUND:** Randomized controlled trials (RCTs) data demonstrate that sevoflurane postconditioning improves clinical outcomes of liver resection with inflow occlusion, presumably due to hepatocyte protection from ischemic injury. However, mechanisms remain unclear. This study examines liver biopsy samples obtained in an RCT of sevoflurane postconditioning to test the hypothesis that sevoflurane attenuates hepatocyte apoptosis.

**METHODS:** Messenger ribonucleic acid (mRNA) of pro- and antiapoptotic regulators Bax and B-cell lymphoma 2 (Bcl2) was examined in hepatic biopsies obtained during the RCT. Hepatic stellate cells (HSCs) and hepatocytes were exposed to hypoxia/reoxygenation (H/R) in vitro to evaluate the effect of sevoflurane postconditioning on apoptosis. The role of HSC as a potential apoptosis trigger in hepatocytes through the production of reactive oxygen species induced by H/R was explored by transferring supernatants from H/R-exposed HSC to hepatocytes as target cells.

**RESULTS:** In patients of the RCT, the Bax/Bcl2 mRNA ratio in liver tissue was markedly decreased in the sevoflurane arm ( $25\% \pm 21\%$  reduction;  $P = .001$ ). In vitro, H/R increased reactive oxygen species production in HSC by  $33\% \pm 16\%$  ( $P = .025$ ), while it was abolished in the presence of sevoflurane ( $P < .001$ ). In hepatocytes, caspase was minimally activated by H/R. However, incubation of hepatocytes with supernatants of HSC, previously exposed to H/R, increased caspase activity by  $28\% \pm 13\%$  ( $P < .001$ ). When exposed to supernatants from HSC undergoing sevoflurane postconditioning, caspase activation in hepatocytes was reduced by  $20\% \pm 9\%$  ( $P < .001$ ), similarly to the sevoflurane effect on the BAX/Bcl2 mRNA ratio in the liver samples.

**CONCLUSIONS:** The study shows that sevoflurane postconditioning affects apoptosis of hepatocytes after ischemia-reperfusion injury in patients. It also demonstrates that HSC may be the effector cells of sevoflurane protection. (Anesth Analg 2018;127:1058–65)

## KEY POINTS

- **Question:** What is the protective mechanism of sevoflurane postconditioning in ischemia-reperfusion injury in the liver? What findings will be expected in vivo analyzed in liver biopsies, based on a randomized control trial, or in vitro in hepatocytes and hepatic stellate cells?
- **Finding:** Pharmacological postconditioning with the volatile anesthetic sevoflurane attenuates ischemic injury-induced apoptosis in hepatocytes through interaction with hepatic stellate cells, which are the injury sensing and transmitting cells through production of reactive oxygen species.
- **Meaning:** An important mechanism of sevoflurane protection in ischemic injury in hepatocytes is an indirect effect of sevoflurane on hepatic stellate cells.

In liver surgery, inflow occlusion (Pringle maneuver) may, at times, be unavoidable to decrease extensive bleeding during resection.<sup>1</sup> Blood loss requiring transfusion is well known to correlate with poor outcomes,<sup>2</sup> including a decrease of cancer-free survival.<sup>3</sup> However, the Pringle maneuver with interruption of oxygen (O<sub>2</sub>) and nutritional

supply induces hepatocyte ischemia-reperfusion (I/R) injury, which is surprisingly well tolerated by the liver but has been shown to cause poor outcomes when used over longer periods of time.<sup>4,5</sup>

Inflow ischemia followed by reperfusion with portal and arterial blood leads to increased levels of aspartate

From the \*Institute of Anesthesiology, University of Zurich, University Hospital Zurich, Zurich, Switzerland; †Institute of Physiology, Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland; ‡Department of Anesthesiology, University of Illinois College of Medicine at Chicago, Chicago, Illinois; §Department of Surgery, Division of Transplant Surgery, Rush University Medical Center, Chicago, Illinois; and ||Department of Surgery, Cantonal Hospital Winterthur, Winterthur, Kanton Zurich, Switzerland. Accepted for publication June 20, 2018.

Funding: This work was supported by the Swiss National Science Foundation grant no. 320030\_141216 (B.B.-S.) and by institutional funding from the University of Zurich (B.B.-S.).

Copyright © 2018 International Anesthesia Research Society  
DOI: 10.1213/ANE.0000000000003692

Conflicts of Interest: See Disclosures at the end of the article.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website ([www.anesthesia-analgesia.org](http://www.anesthesia-analgesia.org)).

This work has been presented as poster at the annual meeting of the International Anesthesia Research Society (IARS) on May 7, 2017 in Washington, DC.

M. Schläpfer and E. Schädde contributed equally and share senior authorship. Reprints will not be available from the authors.

Address correspondence to Beatrice Beck-Schimmer, MD, Institute of Anesthesiology, University Hospital Zurich, Raemistrasse 100, Hof E 111, CH-8091 Zurich, Switzerland. Address e-mail to [beatrice.beckschimmer@uzh.ch](mailto:beatrice.beckschimmer@uzh.ch).

aminotransferase and alanine aminotransferase, which are markers of hepatocyte injury. During inflow ischemia, adenosine triphosphate production decreases with the ultimate consequence of loss of function of the cellular membrane ion pump, swelling of the cell, and, finally, disruption of the cellular membrane structure, a process known as “necrosis.” At the same time, reactive oxygen species (ROS) is produced, which further supports necrosis but also triggers apoptosis, which is a hallmark of I/R injury in hepatocytes.<sup>6</sup> Under normal conditions, proapoptotic Bax and antiapoptotic B-cell lymphoma 2 (Bcl2) proteins in hepatocytes keep an elaborate balance.<sup>7</sup> An increase of the expression of Bax and/or a decrease of Bcl2 enhances downstream activity of caspase-3, which propagates apoptotic cell death. As recently shown, hepatic stellate cells (HSCs) play a crucial role in inflammatory signaling to hepatocytes.<sup>8</sup>

Many studies have been performed to identify protective clinical maneuvers for hepatocytes undergoing I/R injury in the perioperative phase. One approach intervenes during the time period after the injury has just occurred and is called “postconditioning.” Postconditioning was found to be effective to attenuate I/R injury after an ischemic event to myocardium in randomized clinical studies.<sup>9</sup> A similar approach is used in I/R injury to the liver induced by inflow occlusion, applying ischemic or pharmacological postconditioning.<sup>10,11</sup> A randomized controlled trial (RCT) showed the benefit of briefly applying the volatile anesthetic sevoflurane at the beginning of the reperfusion phase.<sup>12</sup> Besides the fact that aspartate aminotransferase and alanine aminotransferase peak values were diminished in the postconditioning group, patients of this arm also experienced fewer complications.<sup>12</sup> While these data suggest a beneficial effect of pharmacological postconditioning, the mechanism of protection is not known.

This study focuses on apoptosis as 1 possible effector pathway of sevoflurane postconditioning in I/R injury, where the process of apoptosis plays a crucial role.<sup>6</sup> Biopsies from an RCT<sup>12</sup> were analyzed. Data were supplemented by experiments using an I/R injury model in hepatocytes in cell culture. It was hypothesized that sevoflurane has an impact on rates of hepatocyte apoptosis. Second, it was postulated that HSCs, but not hepatocytes, mediate the protective effect of sevoflurane.

## METHODS

### Ethics

The study was approved by the institutional review board of the “Kantonale Ethikkommission,” Zurich, Switzerland (Ethical Committee N° StV 34–2007, Chairperson Professor Robert Maurer), on January 14, 2008. Written informed consent was obtained from all subjects.

### Biopsies From Randomized Controlled Study

From January 2008 to September 2010, patients were enrolled in an RCT at the University Hospital Zurich, Switzerland (NCT00518908).<sup>12</sup> The trial was conducted to assess whether pharmacological postconditioning provides hepatocyte protection after inflow occlusion (Pringle maneuver) during liver resection. Patients were randomized into 1 of 3 groups: (1) postconditioning group: propofol anesthesia with inflow occlusion of  $\geq 30$  minutes; on reperfusion, propofol infusion

was stopped, and sevoflurane was flushed in for 5 minutes, followed by exposure to sevoflurane with an end-tidal concentration of 3.2 Vol% for 10 minutes, and then a washout phase of sevoflurane and reinitiating propofol infusion. (2) Intermittent inflow occlusion control arm: propofol anesthesia with inflow occlusion for 15 minutes, followed by a reperfusion time of 5 minutes, which was repeated  $\geq 1$  time to reach a total occlusion time of a minimum of 30 minutes. (3) Continuous inflow occlusion control arm: propofol anesthesia for the entire procedure with a Pringle time of  $\geq 30$  minutes.

In the trial, patient allocation ratio was 3:3:1 based on the sample size calculation. Before inflow occlusion and 45 minutes after reperfusion, a biopsy of liver tissue was obtained, a procedure that had been approved by the Ethic Committee in the protocol of the RCT NCT00518908. Biopsies were immediately snap frozen in liquid nitrogen. In this study, we only evaluated biopsies from patients in the postconditioning and control arms.

### RNA Extraction and Real-Time Polymerase Chain Reaction of Liver Biopsies

Total ribonucleic acid (RNA) from liver biopsies was isolated by homogenization using 1.4-mm ceramic beads (MagNA Lyser Green Beads; Roche Diagnostics Switzerland, Rotkreuz, Switzerland) and a Precellys homogenizer (Bertin Corp, Rockville, MD) for 2  $\times$  30 seconds at 6800 rpm, followed by a purification step with the RNeasy Minikit (Qiagen, Basel, Switzerland). The total amount of RNA was determined at absorbance of 260 nm, purity based on the r260/280 nm ratio using NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, DE). Complementary deoxyribonucleic acid (cDNA) was produced using 0.2- $\mu$ g RNA for the reaction in the cDNA reverse transcription kit (Qiagen, Basel, Switzerland). Quantitative polymerase chain reaction was performed with the help of the FastStart Universal Probe Master Polymerase Chain Reaction Mix (Roche, Basel, Switzerland) with labeled probes (Roche Probe Library, Basel, Switzerland) and respective primers for Bax and Bcl2 (Microsynth, Balgach, Switzerland) (Table). The comparative cycle threshold method was used for quantification of the gene expression. The crossing point values (Secondary Derivative Method; Roche, Basel, Switzerland) of the samples were normalized to the housekeeping gene (18S). Relative messenger ribonucleic acid (mRNA) changes

**Table. Primer and Probe Sequences Used for Real-Time PCR**

Gene	Primer Sequence	Length of Amplicon
Bax		104 nt
Forward	5' ATG TTT TCT GAC GGC AAC TTC 3'	
Reverse	5' ATC AGT TCC GGC ACC TTG 3'	
Probe #57	5' GGC CCC AG 3'	
Bcl2		96 nt
Forward	5' GCA CCT GCA CAC CTG GAT 3'	
Reverse	5' AGC CAG GAG AAA TCA AAC AGA G 3'	
Probe #57	5' ACC TGC TG 3'	
18S		96 nt
Forward	5' GGA GAG GGA GCC TGA GAA AC 3'	
Reverse	5' TGC GGA GTG GGT AAT TTG C 3'	
Probe #74	5' GGC AGC AG 3'	

Abbreviations: Bcl2, B-cell lymphoma 2; PCR, polymerase chain reaction.

of the biopsies after reperfusion compared to the value before reperfusion were calculated for each patient.

### Cell Lines

HSCs (LX-2) were generated by spontaneous immortalization in low-serum conditions.<sup>13</sup> This cell line, a generous gift from Professor Dr Scott Friedman, Mount Sinai School of Medicine, New York, NY, was cultured in Dulbecco's Modified Eagle's Medium high glucose (Gibco, Life Technologies, Zug, Switzerland), 10% fetal bovine serum (Gibco, Life Technologies, Zug, Switzerland), and 1% penicillin/streptomycin (final concentration: 100 U/mL penicillin, 100 µg/mL streptomycin; Gibco, Life Technologies, Zug, Switzerland). When confluence was reached, experiments with HSCs were started.

Hepatocytes (Hep3B), a human hepatoma cell line,<sup>14</sup> were a kind gift of Dr Bruno Stieger, Division of Clinical Pharmacology and Toxicology, University Hospital Zurich, Zurich, Switzerland. Their culture medium consists of Dulbecco's Modified Eagle's Medium with high glucose, 110 mg/L sodium pyruvate, 10% fetal bovine serum, and 1% penicillin/streptomycin. Hepatocytes were seeded at 25,000/mL growth for 2 days until 90% confluence.

### Exposure to Hypoxia/Reoxygenation

In a first approach, confluent hepatocytes were exposed to 0.2% O<sub>2</sub> for 6 hours and reoxygenated in air (21% O<sub>2</sub>) for 24 hours (hypoxia/reoxygenation [H/R]), with or without the presence of 2.2 Vol% sevoflurane in the reoxygenation phase. The lowest O<sub>2</sub> concentration that can be reached in the hypoxic chamber is 0.2%. The constellation of 6-hour hypoxia/24-hour reoxygenation was chosen even not directly comparable to the *in vivo* scenario because lower exposure times do not induce apoptosis according to preliminary finding of our group. In a similar approach, HSCs were exposed to hypoxia for 6 hours and reoxygenated in air (21% O<sub>2</sub>) for a 2- or 24-hour reoxygenation phase with or without the presence of 2.2 Vol% sevoflurane. DNA content and caspase activity were determined in both cell types. Sevoflurane was present during the entire reoxygenation phase (2 or 24 hours).

### DNA Quantitation

The fluorescent dye, bisbenzimidazole H 33258 (Hoechst 33258), is specific for DNA quantitation (range, 10 ng/mL to 10 µg/mL). A DNA quantitation assay with fluorescence measurement was performed using a Tecan reader (excitation of 360 nm, emission at 460 nm) (Sigma, Buchs, Switzerland).<sup>15</sup>

### Caspase Assay

Caspases are aspartate-directed cysteine proteases, which cleave a group of intracellular substrates and thereby contribute to apoptosis. Various physiological and pathological stimuli can activate the inactive precursor proteases. To identify and quantify caspase-3/7/8 activity in apoptotic cells, the fluorogenic tetrapeptide substrate N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) is a useful tool. The fluorogenic 7-amino-4-methylcoumarin (AMC) residue is released by caspase activity, and quantification is performed with the help of a spectrofluorometer (360/465 nm/Tecan infinite M200Pro; Tecan Group Ltd,

Maennedorf, Switzerland). This experimental approach was performed using a commercially available kit (Ac-DEVD-AMC; PeptaNova, Sandhausen, Germany) following the provider's protocol.

### Determination of Intracellular ROS

2',7'-Dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF-DA) (Sigma-Aldrich, Buchs, Switzerland) was used to assess the intracellular levels of ROS. After penetration into the cells, H<sub>2</sub>DCF-DA is hydrolyzed to 2',7'-dichlorodihydrofluorescein by the cellular esterases. In a following step, 2',7'-dichlorodihydrofluorescein is oxidized by intracellular ROS to the highly fluorescent compound 2',7'-dichlorofluorescein.

After a treatment of 6 hours of hypoxia and a short reoxygenation of 2 hours to guarantee detection of ROS, cells were washed and further incubated in a medium containing 50 µM H<sub>2</sub>DCF-DA for 10 minutes. Fluorescence was measured with a microplate spectrofluorometer (Tecan infinite M200Pro), where excitation and emission wavelengths were set to 485 and 528 nm, respectively. As a positive control, Sin-1 was used in a final concentration of 200 µM (Molecular Probes; Thermo Fisher Scientific, Reinach, Switzerland).

### Determination of Hydrogen Peroxide in the Supernatant

Reduction of O<sub>2</sub> produces superoxide, an important component of the group of ROS, which through dismutation forms hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). H<sub>2</sub>O<sub>2</sub> content in supernatants of HSCs was determined using the Amplex (N-acetyl-3,7-dihydroxyphenoxazine) Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen by Thermo Fisher/Life Technologies, Zug, Switzerland). Cells were exposed to hypoxia 0.2% for 6 hours, followed by a 2-hour reoxygenation phase with air, 2.2 Vol% sevoflurane or air/N-acetyl-cysteine (NAC; ROS inhibitor, 10 mM). Eight hours of normoxia was used as the control condition. The Amplex Red Hydrogen Peroxide/Peroxidase technique is widely used to detect extracellular H<sub>2</sub>O<sub>2</sub>. It has high sensitivity and specificity.

H<sub>2</sub>O<sub>2</sub> is of particular interest compared to ROS elements because the former is rather stable. It diffuses actively and freely through the cell membranes, and it can generate locally the hydroxyl radicals by iron-mediated Fenton reaction. The Amplex Red reagent reacts with H<sub>2</sub>O<sub>2</sub> in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin, measured at 540/590 nm.

The supernatants of the cells in different conditions were used (50-µL aliquots were taken from each well of a 96-stripe well plate), and the concentration of H<sub>2</sub>O<sub>2</sub> was measured according to the protocol provided by the manufacturer.

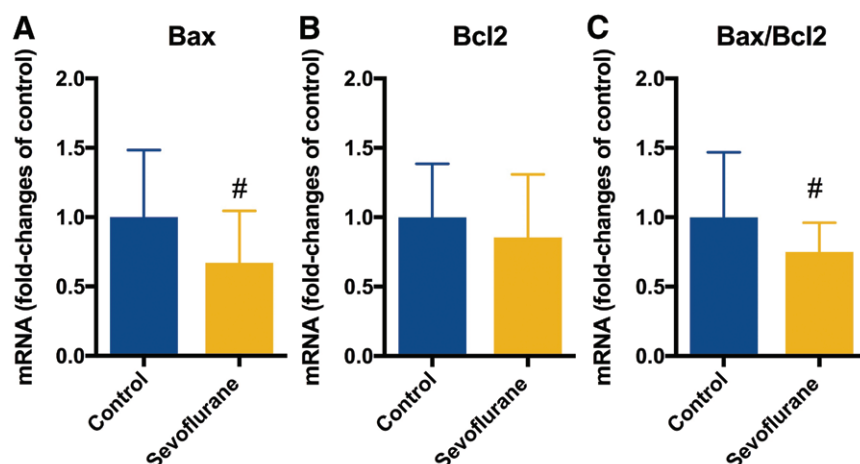
### Use of NAC as Antioxidant (Positive Control for Blocking Step)

HSCs were incubated for 6 hours in hypoxia, followed by a 2-hour reoxygenation with normal air in the presence or absence of the antioxidant NAC (10 mM) (Sigma, Buchs, Switzerland).<sup>16</sup>

### Supernatant Transfer Model

Supernatants from HSCs (H/R 6 hours/2 hours) were removed, centrifuged, and carefully added to a monolayer





**Figure 1.** mRNA expression of Bax and Bcl2 in liver biopsies before and after inflow occlusion. Bax (A) and Bcl2 (B) were determined in liver biopsies from the randomized controlled trial with sevoflurane postconditioning,<sup>12</sup> and their Bax/Bcl2 ratio was calculated (C). Liver tissue, which was collected before the Pringle maneuver, served as baseline (T1). The second biopsy was taken 45 min after initiation of reperfusion (T2). The mRNA changes T2/T1 were calculated and are displayed as fold changes of the control group. Control refers to the arm with patients anesthetized throughout the entire surgery with propofol. In the sevoflurane arm, propofol infusion was stopped at the end of ischemia and replaced by sevoflurane for 10 min (3.2 Vol%).  $n = 17$  in the control group,  $n = 48$  in the sevoflurane group. # $P = .02$  for Bax and Bax/Bcl2. Groups have been compared by an unpaired 2-tailed Student  $t$  test. All data are presented as mean  $\pm$  SD. Bcl2 indicates B-cell lymphoma 2; mRNA, messenger ribonucleic acid.

of hepatocytes for 24 hours. Due to the fast activation of ROS, the 2-hour reoxygenation time point was chosen. DNA and caspase activity were determined in hepatocytes.

### Statistical Analysis

Data are presented as mean  $\pm$  standard deviation. Statistical analyses were performed in GraphPad Prism 6.0 for Mac (GraphPad Inc, La Jolla, CA). Data were tested for normality by Shapiro-Wilks normality test. For the comparison of 2 groups, an unpaired 2-tailed Student  $t$  test of normally distributed data was performed (Figure 1A–C).

For the comparison of  $\geq 3$  groups, a 1-way analysis of variance was performed for normally distributed data. The mean of each group was compared to the mean of the reference group (H/R). A Holm-Sidak post hoc test was performed to correct for multiple comparisons (Figures 2B and 5A).

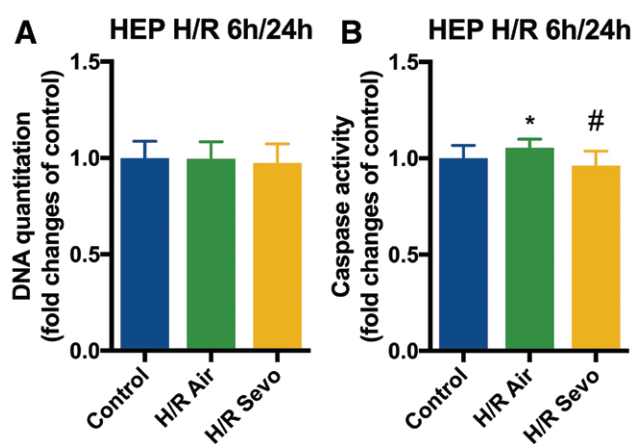
For comparison of  $\geq 3$  groups of not normally distributed data, a Kruskal-Wallis test was performed. The mean rank of each group was compared to the mean rank of the reference group (H/R). A Dunn post hoc test was performed to correct for multiple comparisons (Figures 2A, 3A, 3B, 4A, 4B, and 5B). A flow chart indicates the path of the statistical analyses (Supplemental Digital Content, Figure 1, <http://links.lww.com/AA/C530>). Multiplicity-adjusted  $P$  values are reported and considered significant if  $<.05$ .

At least 3 independent cell experiments were performed with  $\geq 3$  data points per experimental group. The exact number of independent experiments ( $n$ ), the type of statistical tests performed, and adjusted  $P$  values can be found in the figure legend of all of the figures (Figures 1–5).

## RESULTS

### Liver Tissue

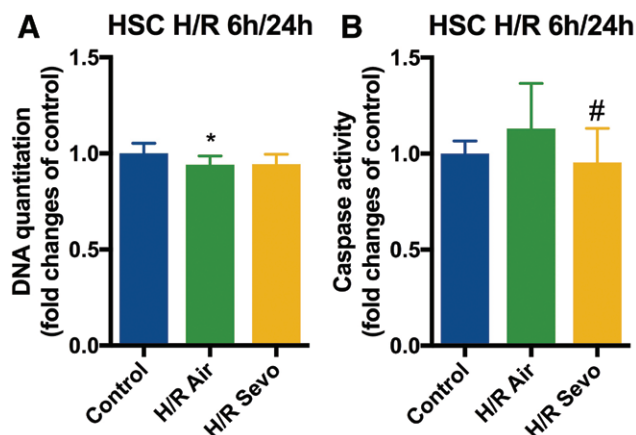
I/R is associated with an increased expression of the proapoptotic regulator Bax, which is attenuated in the presence of sevoflurane postconditioning.



**Figure 2.** Exposure of HEPs to H/R: necrosis and apoptosis. HEPs were grown to confluence and exposed to 6 h of hypoxia (0.2% oxygen) followed by 24 h of reoxygenation in air, with or without the presence of 2.2 Vol% sevoflurane. DNA quantitation was performed (A,  $n = 3$ , Kruskal-Wallis), and caspase activity was determined (B,  $n = 4$ , ANOVA). Data are presented as mean  $\pm$  SD. All values are displayed as proportion of the control mean. \* $P < .001$ , # $P < .001$ . ANOVA indicates analysis of variance; HEP, hepatocyte; H/R, hypoxia/reoxygenation; SD, standard deviation.

Bax and Bcl2 mRNA were measured in liver biopsies of study patients before and 45 minutes after inflow occlusion and compared with the sevoflurane group with postconditioning. At this early time point of reperfusion, determination of these markers seems to be reliable while caspase may not yet be activated.

In the presence of sevoflurane, Bax mRNA was reduced by  $33\% \pm 38\%$  ( $P = .02$ ) (Figure 1A), while Bcl2 mRNA remained unchanged (Figure 1B). As a result, the Bax/Bcl2 mRNA ratio decreased by  $25\% \pm 21\%$  in liver tissue exposed to sevoflurane compared to the control group ( $P = .02$ ) (Figure 1C). This finding suggests less hepatocyte apoptosis in the sevoflurane-treated group, which may explain reduced “transaminitis” in the sevoflurane-treated



**Figure 3.** Exposure of HSCs to H/R: necrosis and apoptosis. HSCs were grown to confluence and exposed to 6 h of hypoxia (0.2% oxygen) followed by 24 h of reoxygenation in air (H/R), with or without the presence of 2.2 Vol% sevoflurane. DNA quantitation was performed (A,  $n = 3$ , Kruskal-Wallis), and caspase activity was determined (B,  $n = 3$ , Kruskal-Wallis). Data are presented as mean  $\pm$  SD. All values are displayed as proportion of the control mean. \* $P = .003$ , # $P = .02$ . H/R indicates hypoxia/reoxygenation; HSC, hepatic stellate cell; SD, standard deviation.

group in the randomized study, leading to decreased peak levels of postoperative transaminases in the blood.<sup>12</sup>

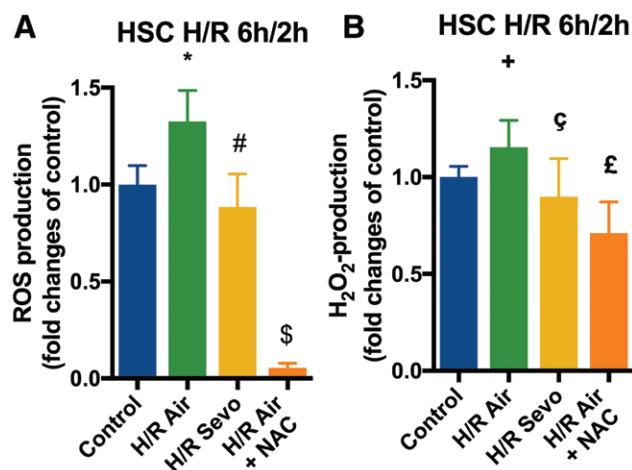
### Hypoxia-Reoxygenation Does Not Directly Trigger Apoptosis of Hepatocytes In Vitro

DNA quantification showed no evidence of hepatocyte cell death after 6 hours of hypoxia and 24 hours of reoxygenation in vitro (Figure 2A), while the apoptosis rate increased by  $5\% \pm 5\%$  ( $P < .001$ ). This increase was fully abrogated in the presence of sevoflurane ( $P < .001$ ) (Figure 2B).

### Hepatic Stellate Cells

**Hypoxia-Reoxygenation Does Not Trigger Apoptosis in These Cells but Induces ROS Production, While Sevoflurane Decreases It.** HSCs are the second most common liver cell type, and they were exposed to H/R injury in vitro. DNA decreased from  $100\% \pm 5\%$  to  $94\% \pm 4\%$  ( $P = .003$ ), and sevoflurane postconditioning had no effect on DNA quantification ( $94\% \pm 5\%$ ) (Figure 3A). Caspase activity was not increased after reoxygenation ( $113\% \pm 23\%$ ;  $P = .07$ ) but was reduced after reoxygenation with sevoflurane compared to reoxygenation with air ( $95\% \pm 18\%$ ;  $P = .02$ ) (Figure 3B).

In the next step, production of ROS and  $H_2O_2$  by HSCs was explored following the hypothesis that ROS and  $H_2O_2$  may play a role in the orchestration of the H/R-induced inflammatory response by mediating injury. Six hours of hypoxia and 2 hours of reoxygenation were chosen because of the fast ROS and  $H_2O_2$  responses on injury. Production of ROS in HSCs increased by  $33\% \pm 16\%$  after H/R injury ( $P = .025$ ) but decreased to baseline after additional postconditioning with sevoflurane ( $P < .001$ ) (Figure 4A). Production of  $H_2O_2$  in HSCs increased by 16% on H/R injury ( $P = .015$ ). This result was attenuated with sevoflurane postconditioning ( $P < .001$ ) (Figure 4B). NAC is a strong antioxidant and was used as a positive control. It completely blunted ROS



**Figure 4.** Exposure of HSCs to H/R: production of ROS. HSCs were grown to confluence and exposed to 6 h of hypoxia (0.2% oxygen) followed by 2 h of reoxygenation in air (H/R), with or without the presence of 2.2 Vol% sevoflurane or NAC as antioxidant (10  $\mu$ M). ROS (A,  $n = 3$ , Kruskal-Wallis) in HSC and  $H_2O_2$  in supernatants of HSC (B,  $n = 5$ , Kruskal-Wallis) were determined. Data are presented as mean  $\pm$  SD. All values are displayed as proportion of the control mean. \* $P = .025$ , # $P < .001$ , \$ $P < .001$ , + $P = .015$ , ç $P < .001$ , £ $P < .001$ . H/R indicates hypoxia/reoxygenation; HSC, hepatic stellate cell;  $H_2O_2$ , hydrogen peroxide; NAC, N-acetyl-cysteine; ROS, reactive oxygen species; SD, standard deviation.

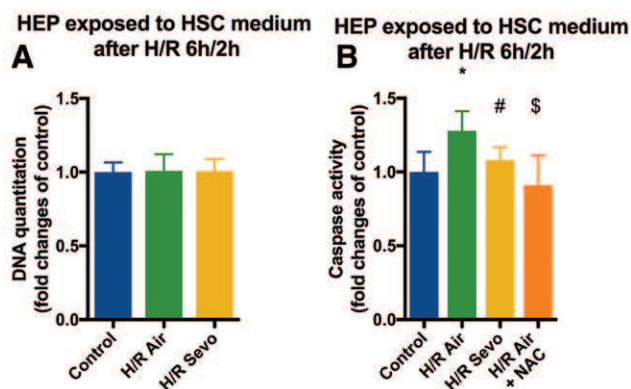
and  $H_2O_2$  production (both  $P < .001$ ), while toxicity was excluded.

### Transfer of HSC Supernatant to Hepatocytes: Supernatant Induces Hepatocyte Apoptosis, Which Is Blunted by Sevoflurane Postconditioning of HSCs

To explore a potentially indirect effect of H/R injury to hepatocytes via HSCs, supernatant of HSCs with H/R injury was then transferred to Hep3B cells and incubated for 24 hours. Hepatocyte cell number did not change (Figure 5A), but caspase activity in hepatocytes exposed to HSC supernatant after H/R increased by  $28\% \pm 13\%$  ( $P < .001$ ) (Figure 5B). Caspase activity in hepatocytes was attenuated by  $20\% \pm 9\%$  when the supernatant was obtained from HSCs postconditioned with sevoflurane ( $P < .001$ ). This decrease is comparable to the sevoflurane-induced attenuation of 25% of apoptosis markers in liver tissue biopsies (Figure 1C). Decreased caspase activity in hepatocytes was also demonstrated after a 24-hour incubation with the supernatant of HSCs previously treated with NAC ( $P < .001$ ) (Figure 5B).

### DISCUSSION

This study combines translational findings from an RCT with an in vitro approach to explore why sevoflurane postconditioning protects from hepatocyte apoptosis after I/R injury to the liver. The in vitro model H/R injury in immortalized liver cell lines suggests that HSCs may transfer injury to hepatocytes by producing ROS, which is known to cause apoptosis of hepatocytes. In vitro, sevoflurane has no direct protective effect on hepatocytes but may have an indirect impact mediated by HSCs. This study presents the first in vitro data about HSCs as effectors of the liver-protective effect of sevoflurane.



**Figure 5.** Exposure of HEPs to supernatant of HSCs previously exposed to H/R: necrosis and apoptosis. HSCs were grown to confluence and exposed to 6 h of hypoxia (0.2% oxygen) followed by 2 h of reoxygenation in air to induce hypoxia-reperfusion (H/R) injury, with or without the presence of 2.2 Vol% sevoflurane or NAC as an antioxidant (10  $\mu$ M). Supernatants were collected and transferred to a confluent layer of HEPs, followed by an incubation of 24 h. DNA quantitation in HEPs was performed (A,  $n = 4$ , ANOVA), and caspase activity was determined (B,  $n = 4$ , Kruskal-Wallis). Data are presented as mean  $\pm$  SD. All values are displayed as proportion of the control mean. \* $P < .001$ , # $P < .001$ , \$ $P < .001$ . ANOVA indicates analysis of variance; H/R, hypoxia/reoxygenation; HEP, hepatocyte; HSC, hepatic stellate cell; NAC, N-acetyl-cysteine; SD, standard deviation.

Pre- or postconditioning with volatile anesthetics is an effective protective strategy to decrease I/R injury to the liver. So far, its efficiency was tested in several models of hepatic I/R in humans<sup>12,17</sup> as well as in vivo,<sup>18,19</sup> showing decreased apoptosis and necrosis rate of hepatocytes. One possible mechanism involved in protection comprises involvement of hepatic energy metabolism.<sup>20</sup> It has been demonstrated in rats that adenosine triphosphate content in the liver significantly decreases in the postischemic phase, which then may be partially restored in the presence of sevoflurane.<sup>20</sup> Another postulated pathway implies the production of nitric oxide, which is higher in the presence of sevoflurane in a model of rat liver transplantation.<sup>21</sup> Schmidt et al<sup>22</sup> showed that isoflurane preconditioning in I/R injury in rats increases the expression of heme oxygenase-1 and emphasized its potential role in the protection provided by volatile anesthetics. These findings were further supported when a heme oxygenase-1 inducer was used instead of isoflurane.<sup>23</sup> The idea that anesthetics modulate the ROS pathway in I/R injury is not new.<sup>24</sup> However, it is generally believed that Kupffer cells and recruited neutrophils are the main source for ROS.<sup>25</sup> In our study, we examined in vitro if HSCs may also be involved in an intercellular cross-talk involving ROS production to induce apoptosis in hepatocytes. While H/R does not induce hepatocytes apoptosis in our cell culture models, it triggers ROS production in HSCs, which in turn causes apoptosis of hepatocytes.

Hepatic I/R injury is a complex orchestration of intercellular and intracellular pathways.<sup>26</sup> The role of Kupffer cells for initiation of I/R injury has been better investigated.<sup>27–29</sup> HSCs, however, are more common cell types in the liver and are constitutively present at the time of injury. Recent literature supports the claims for an important role of HSCs in inflammatory processes.<sup>8</sup> Also, HSC depletion in mice demonstrated an attenuated I/R injury with less production of tumor necrosis factor- $\alpha$  and (C-X-C motif) ligand 1 as well as decreased

infiltration with neutrophils, suggesting that HSCs are not an innocent bystander in inflammatory injury to the liver.<sup>8</sup> Similarly, in a model of pharmacological deactivation of HSCs through an endocannabinoid receptor type 2 agonist in I/R injury in mice, an attenuation of CD4+ T cell infiltration and reduced sinusoidal perfusion failure were observed.<sup>30</sup> Given the complex functionality of HSCs, these findings are not surprising.<sup>31</sup> HSCs are well known as central regulators of the sinusoidal milieu, which requires fast adaptation to injury.<sup>31</sup>

There are several limitations to our study. First, supernatant transfer experiments do not allow direct contact of different cell types. However, it is an elegant method to obtain information about cell interaction without the complexities derived from direct cell–cell contact. The experimental approach is well established, and single-cell type-based assessment of intracellular signaling cascades is possible.<sup>32,33</sup> A second limitation emerges from the limitation of in vitro experiments using established cell lines. While Hep3B cells have hepatoma cell character, LX-2 cells are spontaneously immortalized cells. Apoptosis and ROS production may be altered by virtue of cell line-induced artefacts. A third limitation is that this study does not present in vivo data on the effect size of HSCs in the I/R model. After all, stellate cells have a small cell body with long neuron-like extensions and make up only between 5% and 10% of the hepatic cell mass. The findings reported here need to be validated in an in vivo model of liver I/R injury and hepatocytes apoptosis after HSC depletion. But they may lay the ground for further testing of hypotheses regarding mechanisms of sevoflurane-induced protection in hepatic I/R injury and possible also in other inflammatory models. Also, it should be pointed out that even though there is a strong correlation between sevoflurane conditioning and reduced apoptosis, the causal mechanism is not revealed in our experimental setup.

Fundamentally, the findings presented arose from the observation of increased apoptosis in liver biopsies of patients in the control arm without sevoflurane postconditioning from a randomized cohort of patients with I/R injury.<sup>12</sup> Because an apoptotic effect of H/R on hepatocytes could not be confirmed in vitro, the interest shifted to HSCs and then to the effect of HSCs on hepatocytes. Therefore, supernatant transfer experiments were developed. If these findings can be confirmed, HSCs may be regarded as pharmacological targets of halogenated volatile anesthetics in perioperative medicine.

In conclusion, our data suggest that sevoflurane-induced protection in H/R injury with apoptosis in hepatocytes is mediated through an attenuation of ROS production in HSCs. The role of HSCs and ROS in signaling of I/R injury to the liver has to be further explored. ■■

#### ACKNOWLEDGMENTS

The authors thank Tanja Restin, MD, Institute of Physiology, University of Zurich, Zurich, Switzerland, for her support in the experimental part of the study. The authors also thank Pierre-Alain Clavien, MD, PhD, Visceral and Transplant Surgery, University Hospital Zurich, Zurich, Switzerland, and Stefan Breitenstein, MD, Department of Surgery, Cantonal Hospital Winterthur, Winterthur, Kanton Zurich, Switzerland, for their contribution in designing and performing the clinical trial.



## DISCLOSURES

**Name:** Beatrice Beck-Schimmer, MD.

**Contribution:** This author helped with substantial contribution to conception and design; analysis and interpretation of the data; drafting the article and revising it critically for important intellectual content; and approval of the version to be published.

**Conflicts of Interest:** B. Beck-Schimmer received a grant from Baxter AG, not related to this work. She was a participant of an Advisory Board Meeting of Baxter AG, not related to this topic. She chaired a session (Satellite Symposium on General Anaesthesia and Its Effect on Organ Function: What Do We Know?) at Euroanaesthesia 2013, organized by Baxter AG. She is a councilor of the Swiss National Science Foundation. She is an associate editor of *Anesthesiology*. She received a speaker's fee from Abbvie, Switzerland (pros/cons of volatile anesthetics), for a Grand Round talk in a Swiss Hospital. She has a patent 04/10/14-20140100278: Injectable formulation for treatment and protection of patients having an inflammatory reaction or an ischemia-reperfusion event; M. Urner, L. K. Limbach, I. K. Herrmann, W. J. Stark, and B. Beck-Schimmer applied as PCT (internationally), July 2009.

**Name:** Birgit Roth Z'graggen, PhD.

**Contribution:** This author helped with substantial contribution to design; acquisition and analysis of the data; drafting the article; and approval of the final version to be published.

**Conflicts of Interest:** None.

**Name:** Christa Booy.

**Contribution:** This author helped with substantial contribution to acquisition and interpretation of the data; drafting the article; and approval of the final version to be published.

**Conflicts of Interest:** None.

**Name:** Sabrina Köppel, MMed.

**Contribution:** This author helped with substantial contribution to design and acquisition of the data; revising the article critically for important intellectual content; and approval of the final version to be published.

**Conflicts of Interest:** None.

**Name:** Donat R. Spahn, MD.

**Contribution:** This author helped with substantial contribution to conception and design; interpretation of the data; drafting the article and revising it critically for important intellectual content; and approval of the final version to be published.

**Conflicts of Interest:** D. R. Spahn's academic department is/has been receiving grant support from the Swiss National Science Foundation, Berne, Switzerland; the Ministry of Health (Gesundheitsdirektion) of the Canton of Zurich, Switzerland, for Highly Specialized Medicine; the Swiss Society of Anesthesiology and Reanimation, Berne, Switzerland; the Swiss Foundation for Anesthesia Research, Zurich, Switzerland; Bundesprogramm Chancengleichheit, Berne, Switzerland; CSL Behring, Berne, Switzerland; and Vifor SA, Villars-sur-Glâne, Switzerland. He is the cochair of the ABC-Trauma Faculty, managed by Physicians World Europe, Mannheim, Germany, and sponsored by unrestricted educational grants from Novo Nordisk Health Care AG, Zurich, Switzerland; CSL Behring GmbH, Marburg, Germany; and LFB Biomédicaments, Courtaboeuf Cedex, France. In the past 5 years, he has received honoraria or travel support for consulting or lecturing from the following: Danube University of Krems, Krems an der Donau, Austria; Abbott AG, Baar, Switzerland; AMGEN GmbH, Munich, Germany; AstraZeneca AG, Zug, Switzerland; Baxter AG, Volketswil, Switzerland; Baxter S.p.A., Roma, Italy; Bayer, Zürich, Switzerland, and Berlin, Germany; B. Braun Melsungen AG, Melsungen, Germany; Boehringer Ingelheim (Schweiz) GmbH, Basel, Switzerland; Bristol-Myers-Squibb, Rueil-Malmaison Cedex, France, and Baar, Switzerland; CSL Behring GmbH, Hattersheim am Main, Germany, and Berne, Switzerland; Curacyte AG, Munich, Germany; Daiichi Sankyo (Schweiz) AG, Thalwil, Switzerland; Ethicon Biosurgery, Sommerville, NJ; Fresenius SE, Bad Homburg vor der Höhe, Germany; Galenica AG, Bern, Switzerland (including Vifor SA, Villars-sur-Glâne, Switzerland); GlaxoSmithKline GmbH & Co, KG, Hamburg, Germany; Haemonetics, Braintree, MA; Janssen-Cilag, Baar, Switzerland, and Beerse, Belgium; LFB Biomédicaments, Courtaboeuf Cedex, France; Merck Sharp & Dohme AG, Luzern, Switzerland; Novo Nordisk A/S, Bagsværd, Denmark; Octapharma AG, Lachen, Switzerland; Organon AG,

Pfäffikon/SZ, Switzerland; PAION Deutschland GmbH, Aachen, Germany; Pharmacosmos A/S, Holbaek, Denmark; Photonics Healthcare B.V., Utrecht, the Netherlands; ratiopharm Arzneimittel Vertriebs-GmbH, Vienna, Austria; Roche, Reinach, Switzerland; Sarstedt AG & Co, Sevelen, Switzerland, and Nümbrecht, Germany; Schering-Plough International, Inc, Kenilworth, NJ; Tem International GmbH, Munich, Germany; US Department of Defense, Arlington, Virginia; Verum Diagnostica GmbH, Munich, Germany; and Vifor Pharma, Munich, Germany, Vienna, Austria, and St Gallen, Switzerland.

**Name:** Martin Schläpfer, MD, MSc.

**Contribution:** This author helped with substantial contribution to conception and design; analysis and interpretation of the data; statistical analysis; drafting the article and revising it critically for important intellectual content; and approval of the final version to be published.

**Conflicts of Interest:** M. Schläpfer has received travel honoraria from Baxter AG.

**Name:** Erik Schädde, MD.

**Contribution:** This author helped with substantial contribution to conception and design; analysis and interpretation of the data; drafting the article and revising it critically for important intellectual content; and approval of the final version to be published.

**Conflicts of Interest:** None.

**This manuscript was handled by:** Jean-Francois Pittet, MD.

## REFERENCES

1. Man K, Fan ST, Ng IO, Lo CM, Liu CL, Wong J. Prospective evaluation of Pringle maneuver in hepatectomy for liver tumors by a randomized study. *Ann Surg.* 1997;226:704-711.
2. Kooby DA, Stockman J, Ben-Porat L, et al. Influence of transfusions on perioperative and long-term outcome in patients following hepatic resection for colorectal metastases. *Ann Surg.* 2003;237:860-869.
3. de Boer MT, Molenaar IQ, Porte RJ. Impact of blood loss on outcome after liver resection. *Dig Surg.* 2007;24:259-264.
4. Fellström B, Akütyrek LM, Backman U, Larsson E, Melin J, Zezina L. Postischemic reperfusion injury and allograft arteriosclerosis. *Transplant Proc.* 1998;30:4278-4280.
5. Howard TK, Klintmalm GB, Cofer JB, Husberg BS, Goldstein RM, Gonwa TA. The influence of preservation injury on rejection in the hepatic transplant recipient. *Transplantation.* 1990;49:103-107.
6. Cao L, Quan XB, Zeng WJ, Yang XO, Wang MJ. Mechanism of hepatocyte apoptosis. *J Cell Death.* 2016;9:19-29.
7. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev.* 1999;13:1899-1911.
8. Fujita T, Soontrapa K, Ito Y, et al. Hepatic stellate cells relay inflammation signaling from sinusoids to parenchyma in mouse models of immune-mediated hepatitis. *Hepatology.* 2016;63:1325-1339.
9. Ovize M, Baxter GF, Di Lisa F, et al; Working Group of Cellular Biology of Heart of European Society of Cardiology. Postconditioning and protection from reperfusion injury: where do we stand? Position paper from the Working Group of Cellular Biology of the Heart of the European Society of Cardiology. *Cardiovasc Res.* 2010;87:406-423.
10. Dal Ponte C, Alchera E, Follenzi A, et al. Pharmacological postconditioning protects against hepatic ischemia/reperfusion injury. *Liver Transpl.* 2011;17:474-482.
11. Lin HC, Lee TK, Tsai CC, Lai IR, Lu KS. Ischemic postconditioning protects liver from ischemia-reperfusion injury by modulating mitochondrial permeability transition. *Transplantation.* 2012;93:265-271.
12. Beck-Schimmer B, Breitenstein S, Bonvini JM, et al. Protection of pharmacological postconditioning in liver surgery: results of a prospective randomized controlled trial. *Ann Surg.* 2012;256:837-844.
13. Xu L, Hui AY, Albanis E, et al. Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. *Gut.* 2005;54:142-151.
14. Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science.* 1980;209:497-499.



15. Rengarajan K, Cristol SM, Mehta M, Nickerson JM. Quantifying DNA concentrations using fluorometry: a comparison of fluorophores. *Mol Vis*. 2002;8:416–421.
16. Jiao Y, Ji L, Kuang Y, Yang Q. Cytotoxic effect of oxaloacetate on HepG2-human hepatic carcinoma cells via apoptosis and ROS accumulation. *Neoplasma*. 2017;64:192–198.
17. Beck-Schimmer B, Breitenstein S, Urech S, et al. A randomized controlled trial on pharmacological preconditioning in liver surgery using a volatile anesthetic. *Ann Surg*. 2008;248:909–918.
18. Kon S, Imai M, Inaba H. Isoflurane attenuates early neutrophil-independent hypoxia-reoxygenation injuries in the reperfused liver in fasted rats. *Anesthesiology*. 1997;86:128–136.
19. Rancan L, Huerta L, Cusati G, et al. Sevoflurane prevents liver inflammatory response induced by lung ischemia-reperfusion. *Transplantation*. 2014;98:1151–1157.
20. Bedirli N, Ofluoglu E, Kerem M, et al. Hepatic energy metabolism and the differential protective effects of sevoflurane and isoflurane anesthesia in a rat hepatic ischemia-reperfusion injury model. *Anesth Analg*. 2008;106:830–837.
21. Dal Molin SZ, Krueh CR, de Fraga RS, Alboim C, de Oliveira JR, Alvares-da-Silva MR. Differential protective effects of anaesthesia with sevoflurane or isoflurane: an animal experimental model simulating liver transplantation. *Eur J Anaesthesiol*. 2014;31:695–700.
22. Schmidt R, Tritschler E, Hoetzel A, et al. Heme oxygenase-1 induction by the clinically used anesthetic isoflurane protects rat livers from ischemia/reperfusion injury. *Ann Surg*. 2007;245:931–942.
23. Lv X, Yang L, Tao K, et al. Isoflurane preconditioning at clinically relevant doses induce protective effects of heme oxygenase-1 on hepatic ischemia reperfusion in rats. *BMC Gastroenterol*. 2011;11:31.
24. Xu Z, Yu J, Wu J, et al. The effects of two anesthetics, propofol and sevoflurane, on liver ischemia/reperfusion injury. *Cell Physiol Biochem*. 2016;38:1631–1642.
25. Jaeschke H, Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. *Am J Physiol*. 1991;260:G355–G362.
26. Weigand K, Brost S, Steinebrunner N, Büchler M, Schemmer P, Müller M. Ischemia/reperfusion injury in liver surgery and transplantation: pathophysiology. *HPB Surg*. 2012;2012:176723.
27. Imamura H, Sutto F, Brault A, Huet PM. Role of Kupffer cells in cold ischemia/reperfusion injury of rat liver. *Gastroenterology*. 1995;109:189–197.
28. Jaeschke H. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. *Am J Physiol Gastrointest Liver Physiol*. 2003;284:G15–G26.
29. Jaeschke H, Bajt ML. Regulation of apoptotic signaling pathways in hepatocytes in vivo. *Hepatology*. 2003;37:942–945.
30. Reifart J, Rentsch M, Mende K, et al. Modulating CD4+ T cell migration in the postischemic liver: hepatic stellate cells as new therapeutic target? *Transplantation*. 2015;99:41–47.
31. Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev*. 2008;88:125–172.
32. Bárcena C, Stefanovic M, Tutusaus A, et al. Angiogenin secretion from hepatoma cells activates hepatic stellate cells to amplify a self-sustained cycle promoting liver cancer. *Sci Rep*. 2015;5:7916.
33. Yu G, Jing Y, Kou X, et al. Hepatic stellate cells secreted hepatocyte growth factor contributes to the chemoresistance of hepatocellular carcinoma. *PLoS One*. 2013;8:e73312.